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Measuring Fifteen Endogenous Estrogens Simultaneously in Human Urine by High-Performance Liquid Chromatography-Mass Spectrometry

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A sensitive, specific, accurate, and precise high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry method for measuring the absolute quantities of 15 endogenous estrogens and their metabolites in human urine has been developed and validated. The method requires a single hydrolysis/extraction/derivatization step and only 0.5 mL of urine, yet is capable of simultaneously quantifying estrone and its 2-, 4-methoxy and 2-, 4-, and 16α-hydroxy derivatives, and 2-hydroxyestrone-3-methyl ether; estradiol and its 2-, 4-methoxy and 2-, 16α-hydroxy derivatives, 16-epiestriol, 17-epiestriol, and 16-ketoestradiol in pre- and postmenopausal women as well as men. Standard curves are linear over a 10^3-fold concentration range with the standard error of the estimate (SEE) and the relative standard error of the estimate (RSEE) for the linear regression line ranging from 0.0131 to 0.1760 and 1.2 to 7.3%, respectively. The lower limit of quantitation for each estrogen is 0.02 ng/0.5 mL urine sample (2 pg on column), with the percent recovery of a known added amount of compound (accuracy) of 96−107% and an overall precision, including the hydrolysis, extraction, and derivatization steps, of 1−5% relative standard deviation (RSD) for samples prepared concurrently and 1−12% RSD for samples prepared in separate batches. Since individual patterns of estrogen metabolism may influence the risk of breast cancer, accurate, precise, and specific measurement of endogenous estrogen metabolites in biological matrixes will facilitate future research on breast cancer prevention, screening, and treatment.

The evidence that endogenous estrogens play a role in the development of breast cancer is substantial.[1] Increased breast cancer risk has been reported in women with high circulating and urinary estrogen levels, as well as in those exposed to increased estrogens over time as a result of early onset of menstruation, late menopause, postmenopausal obesity, and/or postmenopausal use of hormone replacement therapy.[2−4] Although the exact mechanism is not fully elucidated, there are two leading hypotheses regarding the role of estrogens in breast carcinogenesis. One of these hypotheses involves catechol estrogens, mainly 2-hydroxyestrone, 2-hydroxyestradiol, and 4-hydroxyestrone (Figure 1), reacting with DNA to form both stable and depurinating adducts and causing other types of oxidative DNA damage that can lead to cell transformation and cancer initiation.[5,6] Alternatively, it has been proposed that the potent mitogenic effects of estrogen are key mechanisms leading to carcinogenesis.[7] In this hypothesis, the 16α-hydroxylated estrogens, such as 16α-hydroxyestrone (Figure 1), would be responsible for breast carcinogenesis due to their much stronger hormonal and mitogenic activity as compared to the catechol estrogens.[7] It is conceivable that quantitatively comparing the levels of endogenous estrogen metabolites in humans who ultimately develop breast cancer to matched, healthy controls could help elucidate the mechanism of breast carcinogenesis and evaluate the risk of developing breast cancer.

Current methods for measuring endogenous estrogen metabolites have involved radioimmunoassay (RIA),[8−11] enzyme immu-

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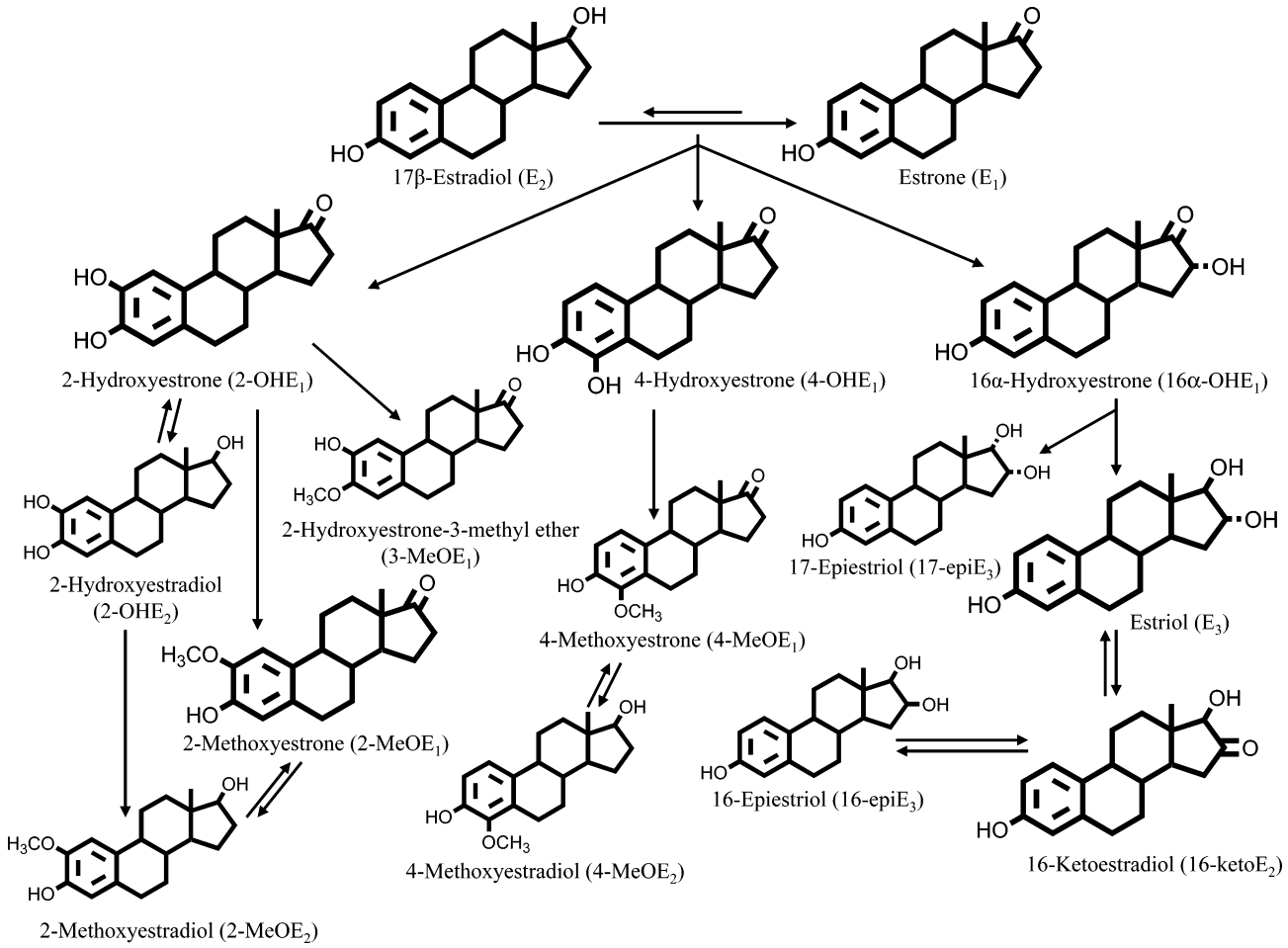

Figure 1. Endogenous estrogen metabolism in humans. The method presented in this paper is capable of quantitatively measuring all of the 15 estrogen metabolites presented above in a single high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry analysis.

noassay (EIA),[12,13] high-performance liquid chromatography (HPLC) with electrochemical detection,[14−16] or stable isotope dilution combined with analysis using gas chromatography/mass spectrometry (GC/MS).[17] Although RIA and EIA can be sensitive, they often suffer from poor specificity, accuracy, and/or reproducibility due to the cross-reactivity and lot-to-lot variation of antibodies.[18−21] Although HPLC with electrochemical detection has been used for estrogen metabolite analysis in hamsters treated with 17β-estradiol[22] and in pregnant women whose estrogen levels are elevated at least 10-fold, it is relatively insensitive.[14−16] Its specificity and accuracy for measuring endogenous level estrogen metabolites in human biological matrixes are questionable. In contrast, the stable isotope dilution GC/MS method is sensitive, specific, and accurate and has been successfully used for urine samples from both nonpregnant premenopausal women and postmenopausal women in which endogenous estrogen metabolites are substantially reduced.[17,23,24] Unfortunately this method is extremely laborious, requiring many steps of solid-phase extractions, ion-exchange column separations, and liquid−liquid extractions, as well as two chemical derivatization procedures for each urine sample.[17,23,24] To overcome this problem, we recently reported methods using HPLC-electrospray ionization (ESI)-MS and ESI-MS^n for measuring endogenous ketolic estrogens and estrogen metabolites in pre- and postmenopausal urine.[25,26] These methods,

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however, are incapable of measuring non-ketolic estrogens and require more than 2.5 mL of urine per sample. In this paper, we present an HPLC-ESI-MS^2 method that requires single hydrolysis, extraction, and derivatization steps and only 0.5 mL of urine, yet is capable of accurately and precisely measuring the absolute quantities of 15 endogenous estrogens and their metabolites, including catechol, methoxy, and 16α-hydroxylated metabolites (Figure 1), found in urines from men as well as pre- and postmenopausal women.

EXPERIMENTAL SECTION

Reagents and Materials. Fifteen estrogen metabolites (EM) including estrone (E_1), estradiol (E_2), estriol (E_3), 16-epiestriol (16-epiE_3), 17-epiestriol (17-epiE_3), 16-ketoestradiol (16-ketoE_2), 16α-hydroxyestrone (16α-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 4-methoxyestrone (4-$MeOE_1$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 4-methoxyestradiol (4-$MeOE_2$), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), and 2-hydroxyestradiol (2-OHE_2) were obtained from Steraloids, Inc. (Newport, RI). Deuterium-labeled estrogen metabolites (d-EM) including estradiol-2,4,16,16-d_4 (d_4-E_2), estriol-2,4,17-d_3 (d_3-E_3), 2-hydroxyestradiol-1,4,16,16,17-d_5 (d_5-2-OHE_2), and 2-methoxyestradiol-1,4,16,16,17-d_5 (d_5-2-$MeOE_2$) were purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). 16-Epiestriol-2,4,16-d_3 (d_3-16-epiE_3) was obtained from Medical Isotopes, Inc. (Pelham, NH). All EM and d-EM analytical standards have reported chemical and isotopic purity ≥98% and were used without further purification. Dichloromethane (HPLC grade), methanol (HPLC grade), and formic acid (reagent grade) were obtained from EM Science (Gibbstown, NJ). Glacial acetic acid (HPLC grade), sodium bicarbonate (reagent grade), and L-ascorbic acid (reagent grade) were purchased from J. T. Baker (Phillipsburg, NJ); sodium hydroxide (reagent grade) and sodium acetate (reagent grade) were purchased from Fisher Scientific (Fair Lawn, NJ). β-Glucuronidase/sulfatase from *Helix pomatia* (Type H-2) was obtained from Sigma Chemical Co. (St. Louis, MO); dansyl chloride (reagent grade), *p*-toluenesulfonhydrazide (reagent grade), and acetone (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Urine Sample Collection. First-morning urine samples were collected in 1 L bottles containing 1 g of ascorbic acid (to prevent oxidation) from 10 premenopausal women (aged from 28 to 47 years, average 33.7 years), 10 postmenopausal women (aged from 53 to 69 years, average 58.7 years), and 5 men (aged from 30 to 39 years, average 32.8 years). All subjects were healthy and nonpregnant, and none of them was taking exogenous hormones. The urine samples obtained from the premenopausal women were collected during both follicular and luteal phase of the menstrual cycle. Immediately after collection, the volumes of the urine samples were recorded. Aliquots of urines were stored at −80 °C prior to analysis. The protocol for this study was reviewed and approved by the NCI/NIH Institutional Review Board.

Preparation of Stock and Working Standard Solutions. Stock solutions of EM and d-EM were each prepared at 80 μg/mL by dissolving 2 mg of the estrogen powders in methanol to a final volume of 25 mL in a volumetric flask and stored at −20 °C. The standard solutions are stable for a minimum of 2 months. No stock solutions older than 2 months were used in the analysis. At the beginning of each analysis, samples of the stock solutions were analyzed to verify that EM and d-EM standards gave the same results as when they were freshly prepared. The 80 ng/mL EM and d-EM working standards were prepared by dilutions of the stock solutions using methanol with 0.1% L-ascorbic acid.

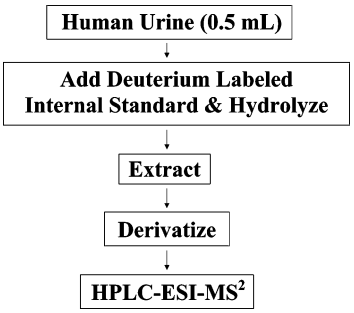

Figure 2. Summary of method for the analysis of 15 endogenous estrogens and their metabolites in urines by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry.

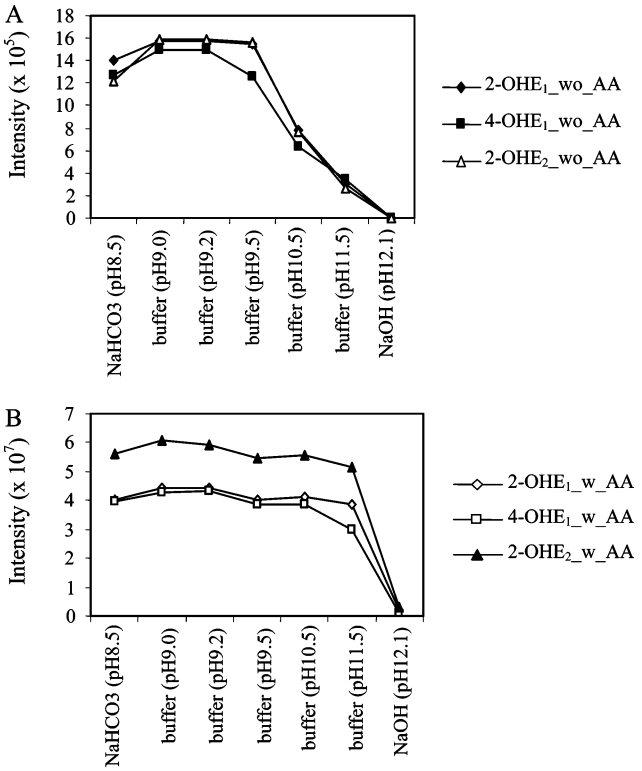

Figure 3. Protection of catechol estrogens by L-ascorbic acid during dansyl derivatization especially when subject to high pH. The same amounts of catechol estrogens were subject to dansylation at increasing pH without L-ascorbic acid (A) or with L-ascorbic acid (B).

Preparation of Calibration Standards and Quality Control Samples. Charcoal stripped human urine (Golden West Biologicals, Temecula, CA) that contains 0.1% (w/v) L-ascorbic acid and has no detectable levels of EM was employed for preparation of calibration standards and quality control samples. Calibration standards were prepared in charcoal stripped human urine by adding 20 μL of the d-EM working internal standard solution (1.6 ng of d-EM) and various volumes of EM working standard solution, which typically contained from 0.02 to 19.2 ng of EM. Quality control samples were also prepared in charcoal stripped human urine at three levels (0.12, 0.96, and 6.4 ng of EM/mL).

Hydrolysis, Extraction, and Derivatization Procedures. The overall procedure for the measurement of EM is shown

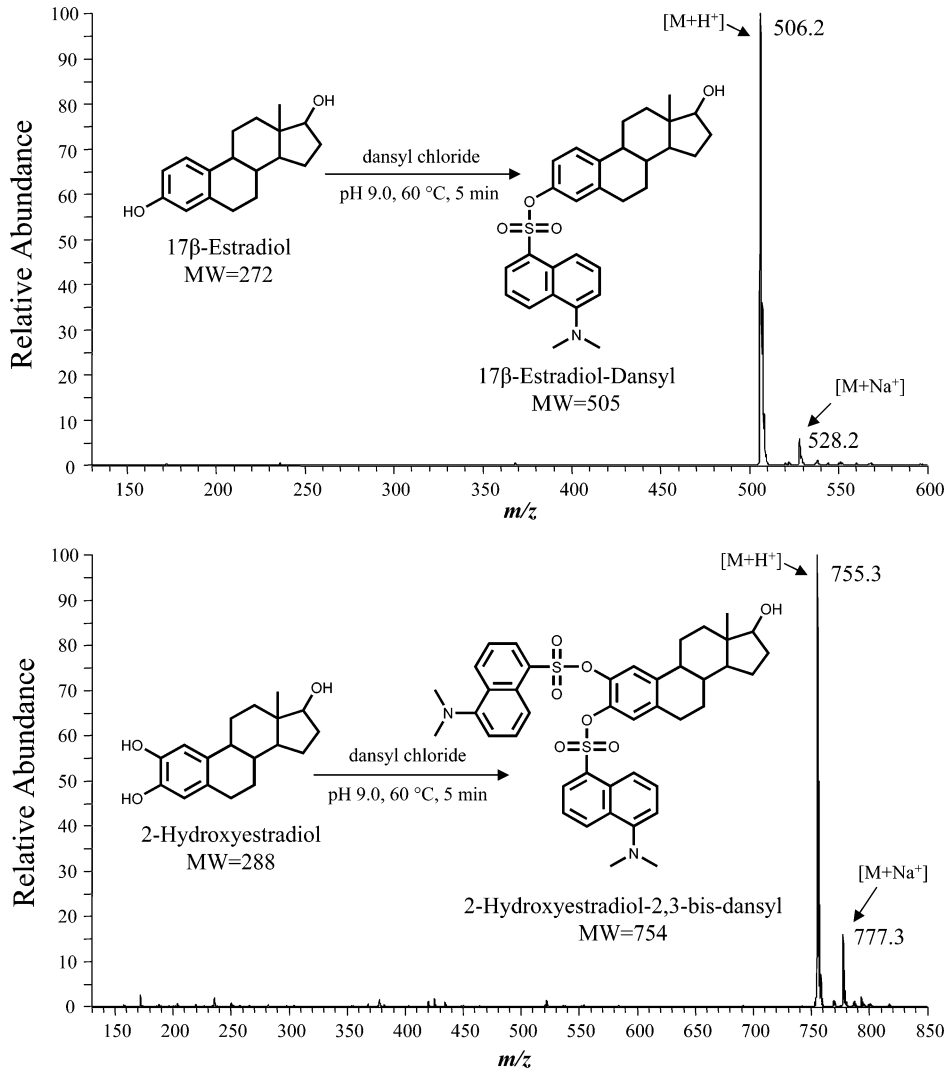

Figure 4. Example mass spectra showing the measurement of the dansylated derivates of 17β-estradiol (A) and 2-hydroxyestradiol (B).

schematically in Figure 2. Since endogenous estrogens and their metabolites in urine are mostly present as glucuronide conjugates and small amounts of sulfate conjugates,[17] a hydrolysis step was included. To a 0.5 mL aliquot of urine, 20 μL of the d-EM working internal standard solution (1.6 ng of d-EM) was added, followed by 0.5 mL of freshly prepared enzymatic hydrolysis buffer containing 2 mg of L-ascorbic acid, 5 μL of β-glucuronidase/sulfatase from *Helix pomatia* (Type H-2), and 0.5 mL of 0.15 M sodium acetate buffer (pH 4.1). The sample was incubated overnight at 37 °C. After hydrolysis, the sample underwent slow inverse extraction at 8 rpm (RKVSD, ATR, Inc., Laurel, MD) with 7 mL of dichloromethane for 30 min. After extraction, the aqueous layer was discarded, and the organic solvent portion was transferred into a clean 16 × 125 mm glass tube and evaporated to dryness at 55 °C under nitrogen gas (Reacti-Vap III, Pierce, Rockford, IL).

To the dried sample, 100 μL of 0.1 M sodium bicarbonate buffer (pH at 9.0) and 100 μL of dansyl chloride solution (1 mg/mL in acetone) were added. After vortexing, the sample was heated at 60 °C (Reacti-Therm III Heating Module, Pierce, Rockford, IL) for 5 min to form the EM and d-EM dansyl derivatives (EM-Dansyl and d-EM-Dansyl, respectively). Calibration standards and quality control samples were hydrolyzed, extracted, and derivatized following the same procedure as that of unknown urine samples. After derivatization, all samples were analyzed by HPLC-ESI-MS^2 (Figure 2).

High-Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry Analysis (HPLC-ESI-MS^2). HPLC-ESI-MS^2 analysis was performed using a Finnigan TSQ Quantum-AM triple quadrupole mass spectrometer coupled with a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Both the HPLC and mass spectrometer were controlled by Xcalibur software (ThermoFinnigan). Liquid chromatography was carried out on a 150 mm long × 2.0 mm i.d. column packed with 4 μm of Synergi Hydro-RP particles (Phenomenex, Torrance, CA) maintained at 40 °C. A total of 20 μL of each sample was injected onto the column. The mobile phase, operating at a flow rate of 200 μL/min, consisted of methanol as solvent A and 0.1% (v/v) formic acid in water as solvent B. For the analysis of EM-Dansyl and d-EM-Dansyl, a linear gradient changing the A/B solvent ratio from 72:28 to 85:18 in 75 min was employed. After washing with 100% A for 12 min, the column was re-equilibrated with a mobile phase composition A/B of 72:28 for 13 min prior to the next injection. The general MS conditions were as follows: source, ESI; ion polarity, positive; spray voltage, 4600 V; sheath and auxiliary gas, nitrogen; sheath gas pressure, 49 arbitrary units; auxiliary

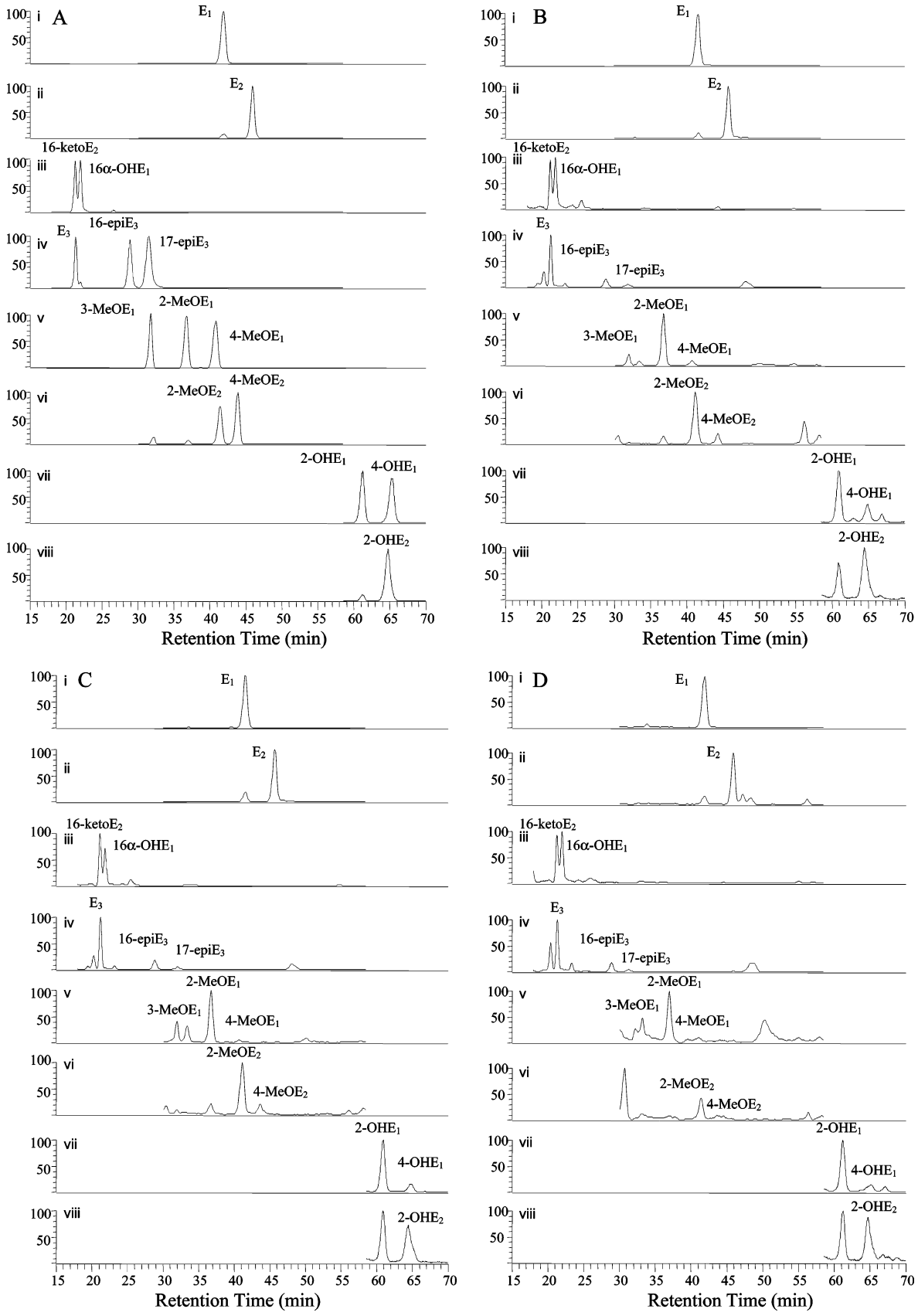

Figure 5. High-performance liquid chromatography-electrospray ionization-tandem mass spectrometry selected reaction monitoring (SRM) chromatographic profiles of dansylated derivates of estrogen and its metabolites corresponding to (A) a 0.12 ng EM/mL urine quality control sample, (B) a 0.5 mL pooled premenopausal urine sample, (C) a 0.5 mL pooled postmenopausal urine sample, and (D) a 0.5 mL pooled male urine sample. (i) E_1; (ii) E_2; (iii) 16-ketoE$_2$ and 16α-OHE$_1$; (iv) E_3, 16-epiE$_3$, and 17-epiE$_3$; (v) 3-MeOE$_1$, 2-MeOE$_1$, and 4-MeOE$_1$; (vi) 2-MeOE$_2$ and 4-MeOE$_2$; (vii) 2-OHE$_1$ and 4-OHE$_1$; and (viii) 2-OHE$_2$.

gas pressure, 23 arbitrary units; ion transfer capillary temperature, 350 °C; scan type, selected reaction monitoring (SRM); collision gas, argon; collision gas pressure, 1.5 mTorr. The SRM conditions for the protonated molecules [MH^+] of EM-Dansyl and d-EM-Dansyl were as follows: E_1 m/z 504 → 171 collision energy: 42 eV; E_2 m/z 506 → 171 collision energy: 43 eV; E_3, 16-epi E_3, and 17-epi E_3 m/z 522 → 171 collision energy: 43 eV; 16-ketoE_2, and 16α-OHE_1 m/z 520 → 171 collision energy: 43 eV; 2-$MeOE_1$, 4-$MeOE_1$, and 3-$MeOE_1$ m/z 534 → 171 collision energy: 42 eV; 2-$MeOE_2$ and 4-$MeOE_2$ m/z 536 → 171 collision energy: 43 eV; 2-OHE_1 and 4-OHE_1 m/z 753 → 170 collision energy: 44 eV; 2-OHE_2 m/z 755 → 170 collision energy: 43 eV; d_4-E_2 m/z 510 → 171 collision energy: 43 eV; d_3-E_3 and d_3-16-epiE_3 m/z 525 → 171 collision energy: 43 eV; d_5-2-$MeOE_2$ m/z 541 → 171 collision energy: 43 eV; d_5-2-OHE_2 m/z 760 → 170 collision energy: 43 eV. The following MS parameters were used for all experiments: scan width, 0.7 u; scan time, 0.50 s; Q1 peak width, 0.70 u fwhm; Q3 peak width, 0.70 u fwhm.

Quantitation of Estrogen Metabolites (EM). Quantitation of EM in urine was carried out using Xcalibur Quan Browser (ThermoFinnigan). Calibration curves for the 15 EM were constructed by plotting EM-Dansyl/d-EM-Dansyl peak area ratios obtained from calibration standards versus amounts of EM and fitting these data using linear regression with $1/X$ weighting. The amount of EM in urine samples was then interpolated using this linear function. The quality of the linear regression line was determined using the standard error of the estimate (SEE) and the relative standard error of the estimate (RSEE).[27] Since deuteriums at the α-position to the carbonyl group of the labeled ketolic estrogens are especially susceptible to exchange loss, deuterium-labeled standards that exhibit no exchange loss were employed in this study. On the basis of their structural similarity and retention times d_4-E_2 was used as the internal standard for E_2 and E_1; d_3-E_3 for E_3, 16-ketoE_2, and 16α-OHE_1; d_3-16-epiE_3 for 16-epiE_3 and 17-epiE_3; d_5-2-$MeOE_2$ for 2-$MeOE_2$, 4-$MeOE_2$, 2-$MeOE_1$, 4-$MeOE_1$, and 3-$MeOE_1$; d_5-2-OHE_2 for 2-OHE_2, 2-OHE_1, and 4-OHE_1.

Absolute Recovery of Estrogen Metabolites after Hydrolysis and Extraction Procedure. To one set of six 0.5-mL aliquots of the charcoal stripped human urine, 20 μL of the EM working standard solution (1.6 ng of EM) was added, followed by the hydrolysis and extraction procedures described above. A second set of six 0.5-mL aliquots of the charcoal stripped human urine was treated identically, except that the EM was added *after* the hydrolysis and extraction procedure. Both sets of samples were derivatized and analyzed in consecutive LC-MS analyses. The absolute recovery of EM after the hydrolysis and extraction procedure was calculated by dividing the mean of EM-Dansyl peak area from the second set into that from the first set.

Accuracy and Precision of the Urinary Estrogen Metabolite Analysis. To assess the percent recovery of the known, added amount of EM (accuracy) and precision of our method, four replicated 0.5-mL aliquots of 0.12, 0.96, and 6.4 ng/mL control urine samples were hydrolyzed, extracted, derivatized, and analyzed in four different batches. The accuracy was measured as the percent matching of calculated amount to known amount of EM in control urine samples. The intra- and inter-batch precisions were measured by the percent relative standard deviations.

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Table 1. Summary of Linear Regression for Calibration Curves[a]

	slope	intercept	SEE (RSEE)
E_1	0.0033 ± 0.0001	0.0009 ± 0.0031	0.0727 (6.0%)
E_2	0.0055 ± 0.0001	0.0239 ± 0.0317	0.1458 (7.3%)
16α-OHE_1	0.0039 ± 0.0001	0.0422 ± 0.0266	0.0631 (4.3%)
16-ketoE_2	0.0038 ± 0.0000	0.0245 ± 0.0091	0.0214 (3.2%)
E_3	0.0029 ± 0.0000	0.0121 ± 0.0055	0.0131 (1.2%)
16-epiE_3	0.0044 ± 0.0001	0.0261 ± 0.0328	0.0776 (4.7%)
17-epiE_3	0.0058 ± 0.0001	0.0204 ± 0.0279	0.0702 (3.2%)
2-OHE_1	0.0056 ± 0.0001	−0.0524 ± 0.0365	0.0864 (2.7%)
2-OHE_2	0.0027 ± 0.0000	−0.0223 ± 0.0170	0.0401 (4.2%)
4-OHE_1	0.0055 ± 0.0001	−0.0532 ± 0.0303	0.0716 (3.7%)
2-$MeOE_1$	0.0052 ± 0.0001	0.0243 ± 0.0271	0.1587 (4.7%)
2-$MeOE_2$	0.0067 ± 0.0001	−0.0136 ± 0.0244	0.1760 (7.2%)
3-$MeOE_1$	0.0051 ± 0.0001	0.0014 ± 0.0276	0.1603 (7.0%)
4-$MeOE_1$	0.0032 ± 0.0000	−0.0168 ± 0.0262	0.0621 (5.3%)
4-$MeOE_2$	0.0061 ± 0.0001	0.0134 ± 0.0351	0.1538 (6.8%)

[a] Linear regression of calibration curve was characterized by the slope and its 95% confidence interval; the intercept and its 95% confidence interval; the standard error of the estimate (SEE) and the relative standard error of the estimate (RSEE) from four separate batches.

RESULTS AND DISCUSSION

Optimal Conditions for Estrogen Metabolite Dansylation. Since the levels of endogenous estrogens and their metabolites can routinely be in the picograms per milliliter range depending on the sex, age, and menopausal status of the patient, it is important that every stage of the analysis be optimized. Therefore, the effects of reaction heating time and temperature, dansyl chloride concentration, pH, and presence of L-ascorbic acid upon the yield of dansylation starting from the same amount of EM were carefully examined. When other conditions were the same, heating sample at 60 °C for 5 min gave the best yield of dansylation for all EM. Increasing dansyl chloride concentration from 1 to 3 mg/mL did not improve the yield of dansylation under the same conditions. No significant change in the extent of dansylation for non-catechol estrogens at pH 8.5−11.5 in the presence or absence of 0.1% (w/v) L-ascorbic acid was observed. The absence of 0.1% (w/v) L-ascorbic acid did, however, result in a significant decrease in the dansylation efficiency of catechol estrogens (Figure 3). Therefore, 0.1% (w/v) L-ascorbic acid was used in all samples including all calibration standards, quality controls, and unknown human urines.

Mass Spectral and Chromatographic Profiles of Estrogens in Quality Control and Pooled Human Urines. The MS full scans of EM-Dansyl and d-EM-Dansyl are characterized by an intense protonated molecule [MH^+], and a much less abundant sodiated molecule [MNa^+] (Figure 4). The major ion in the [MH^+] product ion full scan is observed at m/z 170 for catechol estrogens and m/z 171 for the remaining estrogens and estrogen metabolites. The HPLC-ESI-MS^2 selected reaction monitoring (SRM) chromatographic profiles of a 0.12 ng of EM/mL urine quality control sample, a pooled premenopausal urine sample, a pooled postmenopausal urine sample, and a pooled male urine sample are shown in Figure 5A−D, respectively. Using a simple methanol−

Table 2. Percent Recovery of the Known, Added Amount (Accuracy), and Intra-Batch Precision of Urinary Estrogen Metabolite Measurement, Including Hydrolysis, Extraction, Derivatization, and LC-MS Steps[a]

	0.12 ng/mL urine		0.96 ng/mL urine		6.4 ng/mL urine	
	accuracy (%)	precision (%)	accuracy (%)	precision (%)	accuracy (%)	precision (%)
E_1	102.9	3.1	99.9	4.8	98.9	1.8
E_2	103.4	3.4	98.9	3.2	97.6	1.9
16α-OHE_1	102.6	4.4	103.2	3.6	106.6	1.6
16-ketoE_2	99.5	5.1	103.0	1.8	106.1	2.9
E_3	104.8	3.8	103.2	2.5	107.5	2.5
16-epiE_3	98.2	4.3	96.2	3.0	102.2	2.9
17-epiE_3	102.6	3.3	95.6	3.1	96.7	1.9
2-OHE_1	105.1	3.7	101.3	3.3	102.6	2.6
2-OHE_2	106.1	3.3	103.3	1.3	102.7	1.3
4-OHE_1	106.2	4.5	100.8	2.9	102.2	2.3
2-$MeOE_1$	102.6	3.0	96.2	4.9	97.2	1.8
2-$MeOE_2$	103.2	2.3	97.2	2.2	96.8	1.2
3-$MeOE_1$	106.4	4.4	99.7	3.4	98.4	2.9
4-$MeOE_1$	101.1	2.1	97.4	3.1	98.3	1.6
4-$MeOE_2$	105.9	3.3	98.2	2.8	99.9	2.5

[a] The percent recovery of the known, added amount (accuracy), was measured as the percent matching of calculated amount to known amount of EM in control urine samples. The intra batch precisions were measured as the percent relative standard deviations.

Table 3. Inter-Batch Precision of Urinary Estrogen Metabolite Measurement, Including Hydrolysis, Extraction, Derivatization, and LC-MS Steps[a]

	0.12 ng/ mL of urine	0.96 ng/ mL of urine	6.4 ng/ mL of urine
E_1	8.0	2.1	1.6
E_2	7.3	5.0	1.7
16α-OHE_1	12.1	6.0	1.1
16-ketoE_2	8.2	5.6	2.1
E_3	7.6	3.4	4.0
16-epiE_3	3.8	3.2	2.5
17-epiE_3	6.2	3.3	0.7
2-OHE_1	5.7	4.5	4.6
2-OHE_2	4.4	1.3	1.3
4-OHE_1	7.7	5.2	2.2
2-$MeOE_1$	6.2	3.5	2.0
2-$MeOE_2$	7.0	3.7	1.9
3-$MeOE_1$	9.0	6.5	1.8
4-$MeOE_1$	5.1	4.7	2.3
4-$MeOE_2$	4.9	1.5	1.8

[a] Inter-batch precisions were measured as the percent relative standard deviations.

water linear gradient, all 15 EM were separated by reversed-phase C_{18} chromatography within a 70-min time range, and gave symmetrical peak shapes, which is essential for making accurate quantitative measurements. Even though only single steps of hydrolysis, extraction, and derivatization, and 0.5 mL of human urine sample was used, our method was adequate to quantitatively measure 15 endogenous estrogens and estrogens metabolites in all of the urine samples, even those obtained from men and postmenopausal women.

Calibration Curve and Limit of Quantitation. An important consideration in the development of any assay is the linearity range and sensitivity of the assay. Although the sheer number of EM concentrations being measured in this study typically span between 0.12 and 6.4 ng/mL between the various types of samples that were analyzed in this study, the calibration curves for the detection of each EM were linear over an even broader range (i.e., approximately 10^3-fold) range of concentrations (0.02−19.2 ng/sample or 0.04−38.4 ng/mL). The SEE and the RSEE for the linear regression line ranged from 0.0131 to 0.1760 and from 1.2 to 7.3%, respectively (Table 1). The confidence intervals of the slope were very tight, and the intercept was essentially zero.

The signal-to-noise (S/N) ratios obtained from the 0.02-ng EM-spiked samples prepared in estrogen-free human urine, calibration standard (representing 2 pg of EM on-column) were greater than 200. More importantly, the percent recovery of the known, added amount, and intra- and inter-batch precision at this EM level was consistently between 90 and 110% and within 5 and 15% RSD, respectively. These results demonstrate an adequate limit of quantitation (LOQ) for measuring endogenous EM in urines from human samples, including those obtained from men and post-menopausal women. A 250 fg of EM on-column limit of detection (LOD) can be readily achieved using this method. Both the LOQ and LOD were calculated using the method described in ref 28.

Absolute Recovery of Estrogen Metabolites after the Hydrolysis and Extraction Procedure. Since the concentrations of endogenous estrogen and its metabolites can range into the picogram per milliliter levels in samples obtained from men and postmenopausal women, it is critical that the sample processing procedure retain a high percentage of the starting material. The absolute recovery of EM after the hydrolysis and extraction procedure was determined by comparing chromatographic peak area of EM-Dansyl in charcoal stripped human urine that had been spiked with EM before and after the hydrolysis and extraction procedure. Using this method, the mean absolute recoveries ranged from 86.3 to 93.6%. This high level of recovery not only optimizes the sensitivity of this technique but also increases its overall precision and accuracy.

Accuracy and Precision of the Urinary EM Analysis. To measure the percent recovery of a known, added amount of EM from a sample and the intra-batch precision of this method, four replicated 0.5-mL aliquots of 0.12, 0.96, and 6.4 ng/mL control urine samples were hydrolyzed, extracted, derivatized, and analyzed by HPLC-ESI-MS^2. As shown in Table 2, the percent

(28) Swartz, M.; Krull, I. S. *Analytical Method Development and Validation*, 1st ed.; Marcel Dekker: New York, 1997.

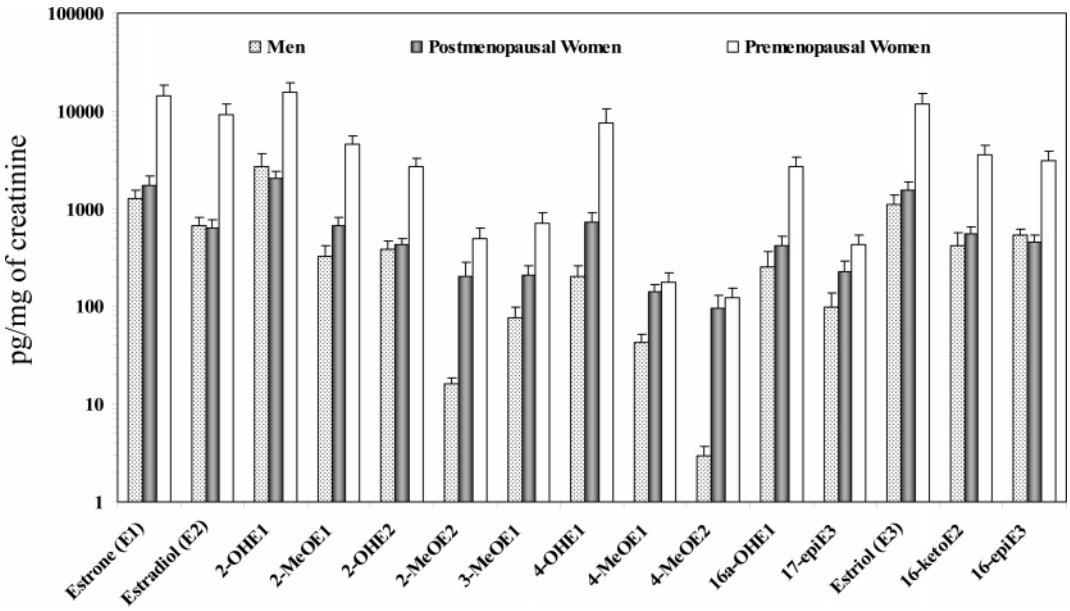

Figure 6. Urinary endogenous EM excretion in postmenopausal women, premenopausal women, and men. Data were expressed as mean and standard error.

recovery of a known added amount of EM from the 0.12, 0.96, and 6.4 ng/mL samples ranged from 98−106%, 96−103%, and 97−107%, respectively (Table 2). The intra-batch precision, as estimated by the RSD from four replicate urine analyses at each concentration level, was 2−5%, 1−5%, and 1−3% for the 0.12, 0.96, and 6.4 ng/mL control urine samples, respectively (Table 2). Inter-batch precision data for the analysis of urinary EM including hydrolysis, extraction, derivatization, and HPLC-ESI-MS^2 steps is presented in Table 3. The inter-batch precision of EM measurement estimated by the RSD from four independent batch analyses ranged from 4−12%, 1−7%, and 1−5% for 0.12, 0.96, and 6.4 ng/mL control urine samples, respectively (Table 3).

Application to Pre- and Postmenopausal and Male Urine Samples. To test its utility for quantitatively measuring estrogen metabolites in actual clinical samples, urine samples from 10 postmenopausal women, 10 premenopausal women, and 5 men were analyzed using the described method. Duplicate 0.5-mL aliquots from each urine sample were hydrolyzed, extracted, derivatized, and analyzed by HPLC-ESI-MS^2 to determine individual EM concentrations (Figure 6). As expected, premenopausal women excreted much greater amount of estrogens and estrogen metabolites than postmenopausal women and men (Figure 6). In addition to the parent estrogens, E_1 and E_2, humans excreted great amount of estrogen metabolites as catechol estrogens such as 2-OHE_1 and 4-OHE_1, from 16α-hydroxylation such as E_3, 16α-OHE_1, and 16-ketoE2, and as methoxy estrogens such as 2-$MeOE_1$ (Figure 6). Significant inter-individual variation has been observed even within the same group such as among postmenopausal women, premenopausal women, or men (Figure 6). Although in most cases the amount of 2-OHE_1 excretion was greater than 4-OHE_1, the opposite was observed in one premenopausal and one postmenopausal woman. Examining the impact of these variations upon breast cancer risk in future epidemiology studies is warranted. This study is the first to provide a detailed measurement of the levels of EM in men (Figure 6). These results suggest the possible use of our method for studying male hormone-related cancers as well.

CONCLUSIONS

With mounting evidence that endogenous estrogens and their metabolites play a role in the development of breast cancer and that women with high circulating and urinary estrogen levels are at an increased risk,[1−4] it is important to develop a sensitive, specific, accurate, and precise assay that can measure individual endogenous estrogens and estrogen metabolites in various biological matrixes. To conclusively determine if a connection between estrogen and estrogen metabolite levels and an increased risk of breast cancer exists requires an epidemiological study in which hundreds of clinical samples could be analyzed. Unfortunately, previously available assays are either too nonspecific or laborious and do not have the capability of measuring the estrogen metabolites that may be of interest.

This manuscript presents a sensitive, specific, accurate, precise, and high-throughput HPLC-ESI-MS^2 method for simultaneously measuring 15 endogenous estrogens and estrogen metabolites in urines from pre- and postmenopausal women and from men. Compared to the previous stable isotope dilution/GC/MS method,[17] our approach greatly simplifies the sample preparation procedure resulting in a high-throughput analytical method that is suitable for epidemiology studies. Standard curves were linear over a 10^3-fold concentration range (0.02−19.2 ng of EM/sample), with the SEE and the RSEE for the linear regression line ranging from 0.0131−0.1760 and 1.2−7.3%, respectively. The lower LOQ for each EM is 0.02 ng/0.5 mL urine sample, with a percent recovery of the a known, added amount of EM of 96−107% and an overall precision of 1−5% RSD for samples prepared concurrently and 1−12% RSD for samples prepared in several batches. We are currently applying this assay in an epidemiology study to determine if there is a link between EM levels and breast cancer risk.

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